SYSTEMATIC SEROLOGY AMONG CERTAIN INSECT SPECIES

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Introduction

The measure of the biochemical similarity among organisms is of interest to all persons doing research whose ultimate goal is to determine the essential nature of organisms. A natural consequence of studies in this direction is the systematic classification of organisms based on the similarities and differences in their serological behavior. The blood sera and the soluble protein constituents have been the chief substrates through which serological classifications have been made. Extractions of whole organisms such as insects have also proved to be adequate for comparing the similarity among insect species (Leone, 1947). Systematic serology does not necessarily answer questions on the phylogenetic origin of organisms, but rather associates animals as they exist today into natural classifications based on their essential biochemical similarities.

The following paper is designed to show the type of serological results that can be expected when various families in the insect order Orthoptera are examined serologically. The organisms used in these tests are listed below:

Family	Scientific name	Common name
	Melanoplus femur-rubrum DeG.	Red-legged grasshopper
	Melanoplus differentialis Thos.	Differential grasshopper
	Romalea microptera Beauv.	Florida lubber grasshopper
Acrididae	*Paroxya atlantica* Scudd.	Atlantic locust
	Leptysma marginicollis Serv.	Slender locust
	Spharagemon bolli Scudd.	Boll's locust
	Arphia xanthoptera Burm.	Yellow-winged locust
Tettigoniidae	*Conocephalus strictus* Scudd.	Straight-lanced grasshopper
	Conocephalus fasciatus DeG.	Slender-meadow grasshopper
Mantidae	*Paratenodera sinensis* Sauss.	Chinese mantis
Gryllidae	*Gryllus assimilis* Fabr.	Field cricket
Blattidae	*Periplaneta americana* (Linn.)	American cockroach

Materials and Methods

Preparation of antigens

After first removing the tarsi and the wings the insects were ground in a mortar with sand. Sufficient buffered 0.85 per cent NaCl was added to prevent drying and denaturation of the saline soluble components. The resulting emulsions were cleared using the same techniques described in a previous paper by the author (Leone, 1947). Micro-Kjeldahl nitrogen determinations were performed to determine the total nitrogen and the non-protein nitrogen. Values for the protein content of the antigens were determined as follows: grams total nitrogen − grams non-protein nitrogen = grams protein nitrogen $\times 6.25 \times 100$ = grams per cent pro-

tein in solution. These values are given in Table I. When only a few insects of a given species were available, it was necessary to dilute the extract sufficiently to obtain volumes large enough to conduct the projected tests. As a result of this the concentrations of protein in solution were markedly reduced in some antigen extracts.

TABLE I

Protein concentrations in extracts of Orthoptera

Organism	Key	Grams protein per cent
Melanoplus femur-rubrum	GR	0.32
Melanoplus differentialis	GD	0.22
Romalea microptera	GF	0.07
Paroxya atlantica	GA	0.05
Leptysma marginicollis	GS	0.03
Spharagemon bolli	GB	0.10
Arphia xanthoptera	GY	0.06
Conocephalus strictus	GL	0.04
Conocephalus fasciatus	GM	0.05
Paratenodera sinensis	MC	0.09
Gryllus assimilis	KC	0.13
Periplaneta americana	CA	0.82

Preparations of antisera

A series of four doubling doses of antigen were injected intravenously in rabbits on alternate days; initial injection was one ml. A second series of antigen injections were given to a rabbit if a preliminary bleeding and testing seven days after the last injection revealed little or no response to the antigen. Final bleedings by cardiac puncture were performed on the eighth day after the last injection. The blood was permitted to clot for 24 hours. The expressed sera were centrifuged, sterile filtered through a Seitz filter, bottled in serum vials, and stored in the refrigerator.

Methods of testing

Ring tests were performed in accordance with standardized procedures as outlined by Boyden (1926).

Photoelectric measurements of precipitin turbidities were made using the Libby photronreflectometer (photron'er) (1938). Titrations were performed and results plotted using the technique of Boyden and DeFalco (1943).

EXPERIMENTAL RESULTS

The production of immune sera against all the Orthoptera except the Blattidae (roaches) proved to be difficult. Protein concentrations were low, and this fact, together with the small quantities of material available restricted the number of injections that could be used to produce an antiserum, and also restricted the number of tests that could be carried out with any one antigen. The photron'er curves proved to have low peaks and broad bases. Inter-family relationships could be established with these curves. Ring test reactions, in general, confirmed the whole curve comparisons of the insect antigens, in so far as this limited technique can be

compared with the nephelometric photron'er method. Typical sets of data are presented to show the correspondence obtained by the two methods (Figs. 1 and 2). A summary of all the information obtained using both techniques is presented in Table II.

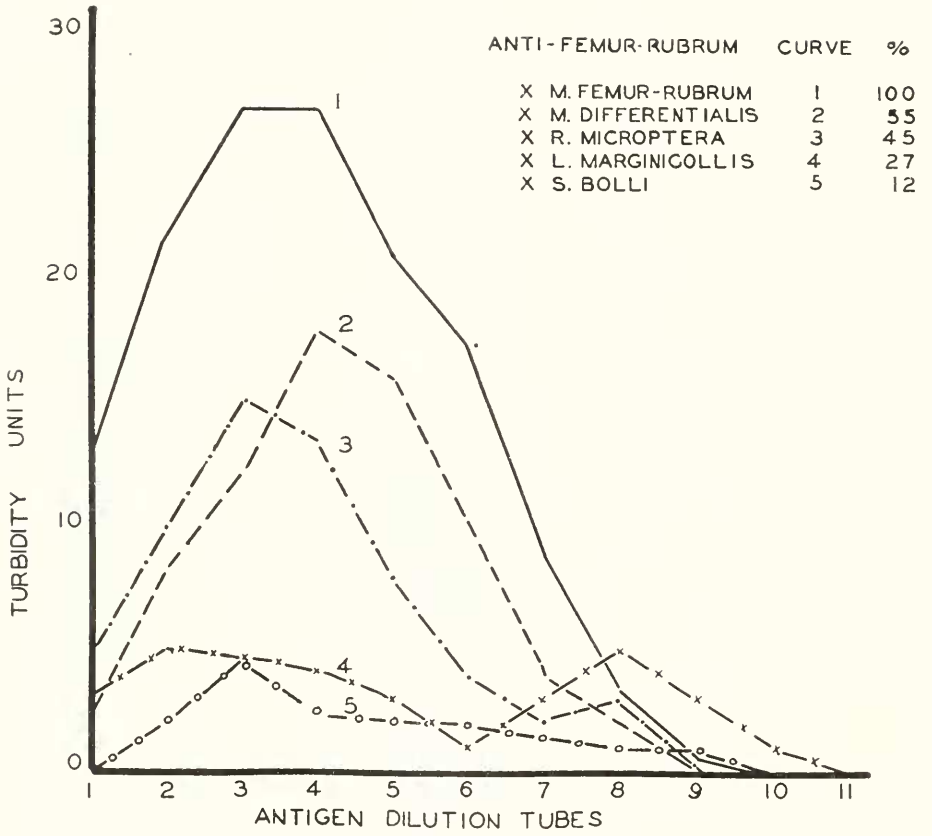


FIGURE 1. A photron'er curve series titrated against an anti-*Melanoplus femur-rubrum* serum. Percentage values indicate the relative degrees of correspondence to the homologous antigen *Melanoplus femur-rubrum*, demonstrated by the various heterologous antigens. The undiluted antiserum shows a low order of turbidities but a broad reactivity range.

Family Acrididae (Short-horned grasshoppers)

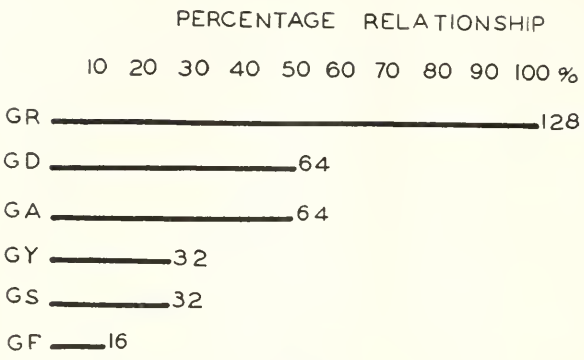
Antisera sufficiently powerful to be recorded on the photron'er were secured in only two instances, one against the Florida Lubber grasshopper (*Romalea microptera*), and the other against the Red-legged grasshopper (*Melanoplus femur-rubrum*). The latter antiserum was obtained as the result of a double series of injections separated by a period of eight days between the first and second injection series.

The anti-Lubber grasshopper serum showed only a slight discrimination between the Red-legged and the Differential grasshoppers (*Melanoplus differentialis*).

TABLE II

Comparison of ring test and photron'er test results

	Antisera	Homologous titer in thousands	Antigens (Relationship values in per cent)											
			Red-legged grasshopper	Differential grasshopper	Florida lubber grasshopper	Atlantic locust	Boll's locust	Yellow-winged locust	Slender locust	Slender-meadow grasshopper	Straight-laced grasshopper	Chinese mantis	Field cricket	American roach
Photron'er Tests	Red-legged grasshopper		100.0	54.9	50.7	44.7		29.2	23.8		12.4	0.0	0.0	0.0
	Florida lubber grasshopper		13.5	9.6	100.0							0.0	0.0	0.0
	Chinese mantis		0.0	0.0	0.0	0.0	0.0	0.0		0.0	0.0	100.0	6.2	18.7
	Field cricket		6.0	4.5			7.3		2.8			10.5	100.0	29.0
	American roach		0.0	0.0				0.0		0.0	0.0	0.0	4.3	100.0
Ring Tests	Red-legged grasshopper	256	100.0	50.0	25.0	50.0		50.0	50.0		12.5	6.8	12.5	6.8
	Differential grasshopper	32	100.0	100.0		50.0								
	Florida lubber grasshopper	64	12.5	3.4	100.0	0.0	0.0	0.0		0.0		0.0	0.0	0.0
	Atlantic locust	32	100.0			100.0	0.0	0.0	0.0			0.0	0.0	0.0
	Chinese mantis	256	0.0	0.0		0.0	0.0	0.0	0.0		25.0	100.0	25.0	25.0
	Field cricket	256	6.8		6.8	0.0	0.0	0.0	0.0		12.5	6.8	100.0	25.0
	American roach	256	6.8	6.8		0.0	0.0	0.0	0.0		12.5	6.8	25.0	100.0
	Red-legged grasshopper (Diluted 1+1)	128	100.0	50.0	12.5	50.0		25.0	25.0		0.0	0.0	0.0	0.0



Key	*Antigens*
GR	Red-legged grasshopper
GD	Differential grasshopper
GA	Atlantic locust
GY	Yellow-winged locust
GS	Slender locust
GL	Straight-lanced grasshopper
GF	Florida lubber grasshopper

FIGURE 2. A typical plot of ring test titers obtained by heterologous antigens titrated against an anti-*Melanoplus femur-rubrum* serum. The number behind each line represents the highest dilution, in thousands, at which a definite interfacial ring was obtained. Ring tests performed using undiluted antiserum gave undifferentiating titers among all the *Acrididae* except the Lubber grasshopper. A dilution of the antiserum with equal parts of saline gave the above discrimination.

The anti-Red-legged grasshopper serum demonstrated an ability to react against many heterologous antigens. Even closely related forms could be readily distinguished, however of the species that showed reactivity, the Straight-lanced grasshopper (*Conocephalus strictus*) belonging to the more distant Tettigoniidae, showed the least amount of turbidity. The mantids, crickets, and roaches produced no heterologous turbidities in the photron'er and thus indicated their more distant relationship.

The confirmation of the reduced reactivity of these more distant species when tested with the ring test was striking. Intra-family differences could not be demonstrated with the ring test (exception may be cited in the case of the Lubber grasshopper *Romalea microptera*). A dilution of one part serum to one part saline reduced the reactivity of the antiserum to such an extent (and also increased its specificity) that all organisms outside the family no longer reacted.

Antisera procured against other members of the family Acrididae, i.e., the Differential grasshopper and the Atlantic locust were too weak to give photron'er readings. Ring test titers for these organisms were very low also, the homologous reactions having a titer of only 1 to 32000.

Family Mantidae (Praying mantids)

Only a single species of this family, the chinese mantis (*Paratenodera sinensis*) was secured. The photron'er results indicate a very specific antiserum with the

families Blattidae and Gryllidae barely making their appearance. The Blattidae (*Periplaneta americana*) show a closer relationship to the Mantidae than any other family considered.

Ring test results indicate undifferentiating heterologous titers among the cricket, roach, and Straight-lanced grasshopper, representing their respective families Gryllidae, Blattidae, and Tettigoniidae when compared against the Mantid. The family Acrididae, evidently more distant, did not react.

Family Gryllidae (Crickets)

In view of the morphological indistinctness of the several large black species of crickets, all the specimens collected were pooled and treated as a single type species (*Gryllus assimilis*) to represent this family. By photron'er test the *Blattidae* (roaches) showed the closest relationship; other families, on the whole, reacted weakly. Greater differentiation of these distant relatives was shown by the ring tests.

Family Tettigoniidae (Long-horned grasshoppers and katydids)

Two species were secured to represent this family, the Straight-lanced grasshopper (*Conoccphalus strictus*) and the Slender-meadow grasshopper (*Conoccphalus fasciatus*). Antisera were not obtained against these antigens. Both species were used however in the heterologous reactions to aid in establishing their position with respect to other species and the position of other species with respect to them.

Family Blattidae (Roaches)

One sample of the American cockroach (*Periplaneta americana*) was chosen to represent this family. The antiserum was the most powerful of all those tested, i.e., gave the greatest turbidity readings, but also was among the most specific of the antisera with respect to inter-family reactions on the photron'er. The field encompassed by means of the ring test technique is somewhat broader than the photron'er examination of this antiserum. The Gryllidae reactions were the most nearly like the Blattidae, of all the families tested. The Tettigoniidae, Acrididae, and the Mantidae showed lessened but approximately the same degrees of relationship to the Blattidae.

Discussion

The results as presented above are at best only a beginning in the study of the problem of the quantitative systematic serology of insects. The insect species used in the tests were chosen because of their availability to the writer. No attempt was made to choose species in such a way, or to perform tests in such a manner as to solve particularly significant problems in insect relationships. It was desired to know the type of serological results that could be obtained using extracts of whole insects as antigens characteristic for the species tested.

The results do indicate the feasibility of making a quantitative serologic analysis of representatives of the families, genera, and species of insects.

Boyden (1943) has been able to establish relatively constant inter-species, inter-generic, and inter-family relationships among the Crustacea. His average value for

the relationship of sera of the same genus is 46 per cent; the averaged value for relationships of genera in the same family is 30 per cent; and, for inter-family relationships the average is 9 per cent.

Intra-generic values for the two species of Melanoplus considered in this paper have a value of 54.7 per cent. The averaged relationships of extracts of genera within the insect family Acrididae proved to have a value of 28 per cent. Inter-family relationships for the order Orthoptera have an average value of 11.5 per cent. The extremes of the serological relationship values for any given group using extracts of fresh insects are all within the extreme values reported by Boyden, who used whole sera for his comparisons. The averaged values obtained for relationships between species, genera, and families are in accord with the Crustacea values.

More information will have to be acquired on the nature of serological reactions, and more data accumulated on actual systematic tests before serological limits in terms of percentages, or other statistical rankings, can be summarized or defined as representing "species," or "genera," or "families" of insects.

Tentative serological relationships for four of the five families of Orthoptera investigated are given in Figure 3. The fifth family tested, the Tettigoniidae are not

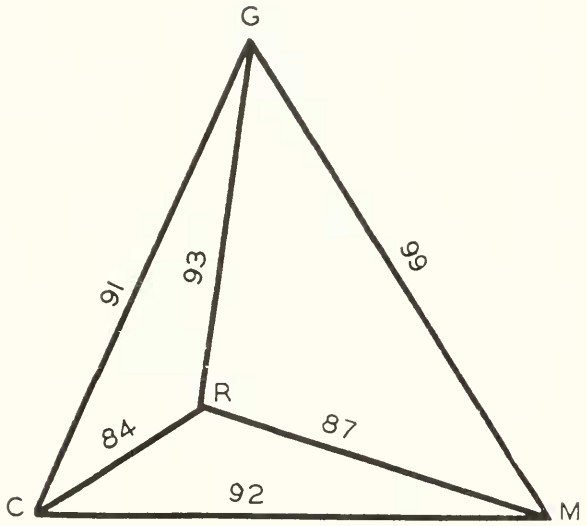


FIGURE 3. A diagram to show the relative distances of four families of Orthoptera from each other. The data are tentative inasmuch as the families have not yet had really adequate testing. The families concerned are Blattidae (*R*), Mantidae (*M*), Gryllidae (*C*), and Acrididae (*G*). The figure requires three dimensions for adequate representation, hence values contiguous to the Blattidae locus are not in proportion with the remainder of the figure. A value of 99 represents the maximum measurable distance between any two families.

represented in Figure 3 inasmuch as no antiserum was produced against this family and reciprocal relationships could not be established. It should be pointed out that the values given are not absolute but only relative. A summary of the relationships of these Orthoptera as determined by the comparison of extracts made from whole insects is as follows:

A. Blattidae—approximately equidistant from the Mantidae and Gryllidae and closer to these families than to the Acrididae.
B. Mantidae—most closely related to the Blattidae. Approximately equally related to the more distant Gryllidae and Acrididae.
C. Gryllidae—closer to Blattidae than to Mantidae and Acrididae.
D. Acrididae—appears to be more closely related to Gryllidae and Blattidae, than to the Mantidae.
E. Tettigoniidae—most distant from the Mantidae and Blattidae of all the families tested. Shows slightly more relationship to the Acrididae than to the other families.

There is striking correlation between the findings of Crampton (1932) in his taxonomic and phylogenetic studies and the orientation of insect families as discussed above, indicating that there may be some correlation between time of origin and degree of similarity in the antigenic constituents of insects, i.e., their serum proteins and other extractable proteins. Except for the position assigned to the Gryllidae, there is also general agreement between the phylogenetic tree of Walker (1922) and the relative positions occupied by the five families of Orthoptera investigated in this paper. As stated previously, however, systematic serology does not attempt to give a final answer to questions on the phylogenetic origin of organisms, but rather associates animals as they exist today into natural classifications based on their essential biochemical similarities. Where serological evidence is in agreement with a paleologic-taxonomic study which correlates phylogenetic origin and present day classifications, then the biochemical (i.e., serological) evidence becomes additive to the other two and increases the likelihood that such a study presents the true picture of the evolutionary development of the species examined.

Summary

1. Representatives of five families of the insect order Orthoptera were compared serologically.

2. On the basis of the degree of serological similarity among them the relative positions of these five families are given.

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